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(54) Bio-organic synthesis of dimeric LeX (difucosyl Y2; III3FucV3-FuncnLc6Cer) and analogues thereof.

(57) A process for preparing difucosyl Y_2 antigen (dimeric Le^x), said process comprising: (1) preparing a lactonorhexaosylceramide backbone or a lactonorhexaosylsaccharide backbone linked to a carrier molecule; and (2) enzymatically fucosylating said backbone at the III^3 and V^3 positions through an α1->3 linkage. A process for preparing Le^y antigen analogues, said process comprising: (1) preparing a lactonorhexaosylceramide backbone or a lactonorhexaosylsaccharide backbone linked to a carrier molecule; (2) enzymatically fucosylating said backbone at the terminal β-Gal through an α1->2 linkage; and (3) enzymatically fucosylating said backbone at one or more positions through an α1->3 linkage, provided that steps (2) and (3) can be conducted simultaneously or in any order. A process for preparing a fucosylated lactonorhexaosylceramide, lactonorhexaosylsaccharide linked to a carrier molecule or higher analogues thereof, said process comprising: (1) preparing a lactonorhexaosylceramide backbone, a lactonorhexaosylsaccharide backbone linked to a carrier molecule or backbones of higher analogues thereof; and (2) enzymatically fucosylating one or more residues of said backbone.

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BIO-ORGANIC SYNTHESIS OF DIMERIC Le^x (DIFUCOSYL Y_2; $III^3FucV^3FucnLc_6Cer$) AND ANALOGUES THEREOF

FIELD OF THE INVENTION

The present invention relates to a process for the synthesis of dimeric Le^x (i.e., difucosyl Y_2; $III^3FucV^3FucnLc_6Cer$) and higher analogues thereof which are cancer cell antigens. Di- or trimeric Le^x is an especially important tumor antigen and is useful for developing vaccines against human cancer and as specific effectors to subside inflammatory processes of rheumatoid arthritis.

More particularly, the invention relates to a process for the synthesis of di- and trifucosyl Le^x and higher analogues by means of a one-step reaction with unexpectedly superior yields.

The present invention also relates to a process for synthesis of Le^y antigen analogues by means of a one-step reaction with unexpectedly superior yields.

BACKGROUND OF THE INVENTION

The antigen dimeric Le^x(difucosyl Y_2; $III^3FucV^3FucnLc_6Cer$) and trimeric Le^x (III^3FucV^3Fuc $VIII^3FucnLc_8Cer$) are the major antigens found in various human adenocarcinomas, and are absent in corresponding normal tissue (Hakomori, S. et al., 1984, J. Biol. Chem., 259, 4672-2680). A monoclonal antibody directed to this structure, but not cross-reacting with simple Le^x (see Table I) is an important reagent for detection of the presence of this antigen in tumor cells (Fukushi, Y. et al., 1984, J. Biol. Chem., 259, 4681-4685; Fukushi, Y. et al. 1984, J. Exp. Med., 159, 506-520) and in sera of patients with cancer. The antibody, however, also reacts on immunoperoxidase or immunofluorescence with a few normal cells such as epithelial cells of proximal convoluted tubules of kidney and weakly with some subpopulations of granulocytes (Fukushi, Y. et al., 1984, Cancer Res., 45, 3711-1717). More recently, the same antigen was found to be expressed in granulocytes of inflammatory bone marrows adjacent to joints affected by rheumatoid arthritis (RA) (Ochi T., et al, 1988, J. Rheumatol., 15, 1609-1615). Intradermal inoculation of this glycolipid antigen included in liposomes suppresses the appearance of inflammatory granulocytes in the bone marrow of RA-affected joints. Subsequently, RA symptoms are greatly reduced. Because of the presence of this antigen in high concentration in various types of human cancer and inflammatory processes, the antigen is expected to be a useful component for developing an anti-cancer and an anti-inflammatory vaccine. To support this idea, this antigen, included in Newcastle's Disease virus membrane (reconstituted virus membrane including the dimeric Le^x antigen) was found to induce immune response to suppress growth of murine tumors bearing the Le^x antigen.

Le^y antigens, including extended Le^y (Le^y octasaccharide ceramide) and trifucosyl Le^y are also important human cancer antigens and are expected to be useful components for developing anti-cancer vaccines.

Thus, demand for dimeric Le^x antigen and various types of Le^y antigen for use in active immunization has been increasing. However, it has been available only from human cancer tissue or via chemical synthesis as described by Nilsson et al. (Nilsson, M., and Norberg, T., 1987, Glycoconjugate J., 4, 219-223; 1988, Carbohydr. Res., 183, 71-82) and Sato et al. (Sato, S., Ito, Y., and Ogawa, T., 1988, Tetrahedron Lett., 29, 5267-5270). Preparation from tumor cells results in limited quantities and the presence of impurities. On the other hand, pure chemical synthesis involves at least 50 steps, is extremely laborious, and results in a poor final yield.

It is toward the objective of providing a low-cost, simplified and greater-yielding synthesis of dimeric or trimeric Le^x antigens and Le^y antigen that the present invention is directed.

SUMMARY OF THE INVENTION

The principal object of the present invention is to provide a low-cost, simplified process for the synthesis of dimeric or trimeric Le^x antigens having improved greater yields.

Another object of the present invention is to provide a low cost, simplified process for the synthesis of

trifucosyl Le^Y (VI^2FucV^3 $FucIII^3FucnLc_6$) or its higher homologs.

These and other objects of the invention have been achieved by providing a process for preparing difucosyl Y_2 antigen (dimeric Le^x), the process comprising: (1) preparing a lactonorhexaosylceramide backbone or a lactonorhexaosylsaccharide backbone linked to a carrier molecule; and (2) enzymatically fucosylating the backbone at the III^3 and V^3 positions through an α1->3 linkage.

The present invention also provides a process for preparing Le^Y antigen analogues, the process comprising:

(1) preparing a lactonorhexaosylceramide backbone or a lactonorhexaosysaccharide backbone linked to a carrier molecule;

(2) enzymatically fucosylating the backbone at the terminal β-Gal through an α1->2 linkage; and

(3) enzymatically fucosylating the backbone at one or more positions through an α1->3 linkage, provided that steps (2) and (3) can be conducted simultaneously or in any order.

The present invention also provides a process for preparing an α1->2 and/or α1->3 fucosylated lactonorhexaosylceramide, lactonorhexaosylsaccharide linked to a carrier molecule or higher homologues thereof, said process comprising: (1) preparing a lactonorhexaosylceramide backbone, a lactonornexaosylsaccharide backbone linked to a carrier molecule or backbones of higher homologues thereof; and (2) enzymatically fucosylating one or more residues of said backbone.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 outlines the syntehtic plan for lactonorhexaosyl structure (**6**) by a stepwise approach (mode I).

Figure 2 outlines the synthetic plan for lactonorhexaosyl structure (**6**) by a stepwise approach (mode II, or convergent approach).

Figure 3 describes a known synthetic route for O-(2,3,4,6-tetraO-acetyl-β- D -galactopyranosyl)-(1->4)-1,3,6-tri-O-acetyl-2-deoxy-2-phthalimido-α (and β)- D -gulocopyranose (**2**).

Figure 4 describes the known transformation of 2 into N-phthaloyl lactosaminyl donors (**3a**, **3b**, and **3c**).

Figure 5 describes the known synthesis of lactosyl acceptors **4a** and **4b**.

Figure 6 describes the entire procedure for synthesis of lactonorhexaosyl structure (**6**) by a stepwise approach (mode I) (detailed scheme).

In Figures 1-6, Gal represents galactose, GlcNAc represents N-acetylglucosamine, Glc represents glucose, Ac represents an acetyl group, Bn represents a benzyl group, Bz represents a benzoyl group, Nphth represents an N-phthalyl group, SMe represents a thiomethyl group, Ac_2O repersents acetic anhydride, Py represents pyridine, DMF represents N,N-dimethylformamide, and MS AW-300 represents molecular sieves AW-300.

Fig. 7 shows the results of subjecting the monosialoganglioside fraction of bovine red blood cells (RBC) to high performance liquid chromatography (HPLC). Fig. 7A shows the elution pattern of the monosialyl gangliosides through an Iatrobead column. The arrow indicates sialylnorhexosyl ceramide. Fig. 7B shows the thin layer chromatograph of fractions 51 to 59. Arrow 1 indicates sialyl lactonoroctaosylceramide and arrow 2 indicates sialyl lactoisooctaosylceramide. Fig. 7C shows the thin layer chromatographs of several pooled fractions. Fig. 7D shows the separation pattern after the pooled fractions 55-58 in Fig. 7C were subjected to a second HPLC.

Fig. 8 shows immunostained thin layer chromatographs of enzymatically synthesized dimeric Le^x. Fig. 8A shows the pattern after staining with orcinol and H_2SO_4, which detects carbohydrate. Fig. 8B shows the pattern after staining with monoclonal antibody SH1, which detects long and short chain Le^x glycolipid. Fig. 8C shows the pattern after staining with monoclonal antibody SH2, which detects only long chain Le^x - (dimeric Le^x). In Figs. 8A, 8B and 8C the lanes are as follows: lane 1, lactonoroctylceramide (nLc_6), standard from bovine RBC; lane 2, enzyme reaction mixture without nLc_6; lane 3, enzyme reaction mixture with nLc_6; lane 4, standard dimeric Le^x from human tumor; lane 5, standard "O" upper neutral from human RBC; lane 6, tumer upper neutral.

Fig. 9 is an autoradiogram showing the results of GDP-^{14}C-fucose incorporation into nLc_4 and nLc_6 from bovine and human placenta lactosyl substrates. Lane A, bovine RBC lactoneotetraosylceramide (nLc_4) and GDP-^{14}C-fucose enzyme reaction mixture; Lane B, human placenta nLc_4 and GDP-^{14}C-fucose enzyme reaction mixture; Lane C, bovine RBC nLc_6 and GDP-^{14}C-fucose enzyme reaction mixture; Lane D, human placenta nLc_6 and GDP-^{14}C-fucose enzyme reaction mixture.

Fig. 10 shows TLC immunostaining of nLc_4 conversion to Le^x. Fig. 10A shows the pattern after staining with orcinol-H_2SO_4. Fig. 10B shows the pattern after staining with anti-Le^x antibody SH1. Fig. 10C shows the pattern after staining with anti-dimeric Le^x antibody SH2. In Figs. 10A, 10B and 10C the lanes are as follows: lane 1, standard "O" upper neutral from human RBC; lane 2, tumor upper neutral; lane 3, tumor Le^x standard; lane 4, enzyme reaction mixture with nLc_4 from human placenta; lane 5, enzyme reaction mixture with nLc_4 from bovine RBC.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have found the following system highly efficient for synthesis of dimeric Le^x antigen (difucosyl Y_2). The backbone structure, i.e., lactonorhexaosyl saccharide or its ceramide conjugate, is syntehsized by organic chemical reaction or easily prepared from bovine red blood cells with high yield. Subsequent α1->3 fucosylation at the III^3 and V^3 positions is carried out by α1->3 fucosyltransferase and GDp-fucose. The enzyme α1->3 fucosyltransferase is found with high activity in the Colo205 human colon cancer cell line, which has no α1->2 fucosyltransferase. Thus, the final product, dimeric Le^x antigen, can be obtained from the backbone structure by a one-step reaction, with 70-80% yield. The overall cost of preparing the antigen in this manner is 1% or less compared to the multi-step chemical synthetic method. Further, the method can be used to prepare fucosylated analogues of the lactonorhexaosyl structure such as lactonoroctaosyl, lactonordecaosyl, or higher homologues (polylactosamines). Examples of compounds, other than the dimeric Le^x antigen, that can be synthesized by the process of the present invention include the Y_2 antigen, the Z_1 antigen, the Z_2 antigen, the Z_3 antigen (trimeric Le^x). The structures of these compounds along with their short chain analogues are shown in Table I below.

Le^y octasaccharide ceramide, trifucosyl Le^y, and Le^y hexasaccoharide ceramide are also important human tumor antigens (Abe et al, 1983, J. Biol. Chem. 258, 11793-11797; Nudelman et al, 1986, J. Biol. Chem. 261, 11247-11253; Kaizu et al, 1986, J. Biol. Chem. 261, 11254-11258), and the structures of these compounds are shown in Table II below. Synthesis of Le^y antigen analogues as in Table II can be made by treating the core carbohydrate chains with α1->2 fucosyltransferase and GDP-fucose followed by α1->3 fucosyltransferase and GDP-fucose, or a combination of these two enzyme reactions. Although a practical experimental example is not given in the application, α1->2 fucosyltransferase can be enriched from colonic cancer cell line MKN74 according to our preliminary experience. Other cell lines that may have higher enzyme activity are also available.

TABLE I

MONO- AND DIMERIC Le^x ANTIGEN AND RELATED STRUCTURES

Le^x pentasaccharide ceramideN

Galß1->4GlcNAcß1->3Galß1->4Glcß1->1Cer
3
↑
Fucα1

Y_2 antigen

Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Galß1->4Glcß1->1Cer
3
↑
Fucα1

difucosyl Y_2 (dimeric Le^x)

Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Galß1->4Glcß1->1Cer
3 3
↑ ↑
Fucα1 Fucα1

Z_1 antigen

Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Gal-
3
↑
Fucα1
ß1->4Glcß1->1Cer

Z_2 antigen

Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Gal-
3 3
↑ ↑
Fucα1 Fucα1
ß1->4Glcß1->1Cer

Z_3 antigen (tr meric Le^x)

Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Gal-
3 3 3
↑ ↑ ↑
Fucα1 Fucα1 Fucα1
ß1->4Glcß1->1Cer

TABLE II

Le^Y ANTIGEN AND ANALOGUES

Le^Y hexasaccharide ceramide ($IV^2FucIII^3FucnLc_4$)

Galß1->4GlcNAcß1->3Galß1->4Glcß1->1Cer
2 3
↑ ↑
Fucα1 Fucα1

Le^Y Octasaccharide ceramide ($VI^2FucV^3FucnLc_6$)

Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Galß1->4Glcß1->1Cer
2 3
↑ ↑
Fucα1 Fucα1

trifucosyl Le^Y ($VI^2FucV^3FucIII^3FucnLc_6$)

Galß1->4GlcNAcß1->3Galß1->4GlcNAcß1->3Galß1->4Glcß1->1Cer
2 3 3
↑ ↑ ↑
Fucα1 Fucα1 Fucα1

Conditions of synthesis for the method of the present invention are described below.

Lactonorhexaosylceramide (Galβ1->4GlcNAcβ1->3-Galβ1->4GlcNAcβ1->3Galβ1->4Glcβ1->1Cer) or its longer chain analogues such as lactonoroctaosyl, lactonordecaosyl, or higher homologues can be prepared as glycolipid from a natural source, e.g., from human placenta, bovine red blood cells, or rabbit muscle. These tissues and cells contain a large quantity of sialyl 2->6 or 2->3 lactonorhexaosylceramide

and longer chain analogues thereof, which can be easily isolated by Folch's partition of total lipid extract followed by DEAE-Sepharose chromatography and high performance liquid chromatography in isopropanol-hexane-water (for detailed procedure see: Hakomori, S. "Chemistry of Glycosphingolipds" In: Handbook of Lipid Research 3: Sphingolipd Biochemistry (Kanfer JN, Hakomori S., eds.), Plenum Publishing, New York, pp. 1-165, 1983). Lactonorhexaosylceramide or its longer chain analogues can be derived from the sialyl counterpart by hydrolysis of the sialic acid with a weak acid (e.g. 1% acetic acid, 80-90 ° C for 1 hr.).

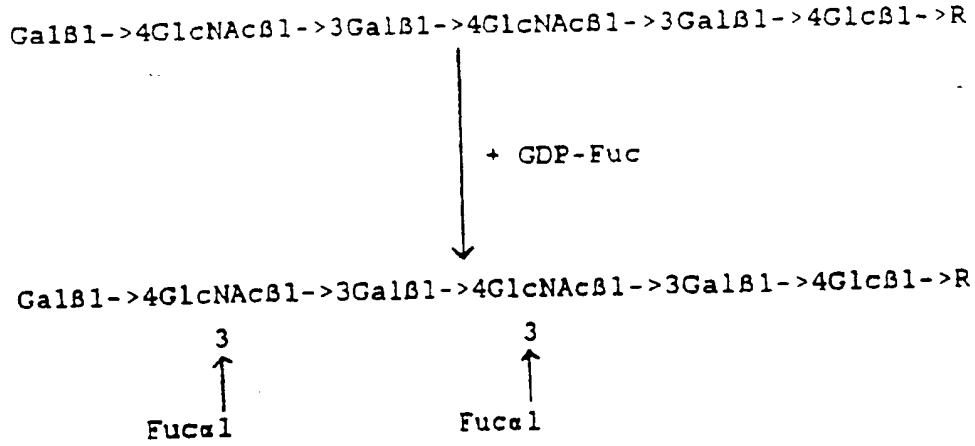
As an alternative to isolation from natural sources, lactonorhexaosylsaccharide or its longer chain analogues linked to carrier molecules such as ceramide or to a protein such as BSA through Lemieux's arm can be chemically synthesized. Such procedures have been described previously (Ogawa, T., 1987, Carbohydr. Res., 163, 189-208; 167, 197-210; Lemieux, R.U., Bundle, D.R. and Baker, D.A., 1975, J. Amer. Chem. Soc., 97, 4076-4083). Various synthetic routes starting from lactose and N-phthalyl-lactosamine are shown in Figures 1 to 6.

Figure 1 shows a synthetic plan for the lactonorhexaosyl backbone **6** by a stepwise approach. According to this plan the hexasaccharide **6** can be synthesized by the coupling of a N-phthalyl lactosaminyl donor (**3a**, **3b**, and **3c**) and a glycotetraosyl acceptor (**5a** and **5b**), which in turn can be synthesized by the coupling of the **3a**, **3b**, or **3c** with glycobiosyl acceptor **4a** or **4b**. The different lactosaminyl donors **3a** (Arnap, J., and Lonngren, J., 1981, J. Chem. Soc. Perkin Trans., 1, 2070-2074), **3b** - (Excoffier, G., Gagnair, D., and Utille, J.P., 1981, Carbohydr. Res., 93, Cl; Grundler, G., and Schmidt, R.R., 1985, Carbohydr. Res., 135, 203-), and **3c** (Sadozai, K.K., Nukada, T., Ito, Y., Nakahara, Y., and Ogawa, T., 1985, Carbohydr. Res., 157, 101-123) can readily be prepared from **2**, which in turn is synthesized from commercially available lactose in seven steps as described in Figure 3 (Haworth, W.N., Hirst, E.L., Plant, M.M.T., and Reynolds, R.J.W., 1930, J. Chem. Soc., 1930, 2644-2653; Arnap, J., and Lonngren, J., 1981, J. Chem. Soc. Perkin Trans., 1, 2070-2074). The lactosyl acceptor **4a**, which has free hydroxyl groups at C-3 and C-4 of the galactose unit (Koike, K., Sugimoto, M., Sato, S., Ito, Y., Nakahara, Y., and Ogawa, T., 1987, Carbohydr. Res., 163, 189-208; Paulsen, H., and Paal, M., 1985, Carbohydr. Res., 137, 39-62) or **4b**, which has a free hydroxyl group only at C-3 of the galactose unit (Yoshino T., Sadamitsu, M., Minagawa, M., Reuter, G., and Schauer, R., 1988, Glycoconjugate J., 5, 377-384) can also be prepared from lactose **1** by the known procedures as described in Figure **4**.

Due to the higher reactivity of the 3'-OH groups, the glycosylation of **3b** with **4a** preferentially gives the tetrasaccharide **7a** with its (1->4) analogue **8a** as a minor product (Figure 6) (Paulsen, H., and Michael, S., 1987, Carbohydr. Res., 169, 105-125; Ito, Y., and Ogawa, T., 1986, Agric. Biol. Chem., 50, 3231-3233). Alternatively, the glycosylation of **3b** with **4b** gives only one tetrasaccharide **7b** in much better yield. Both of the tetrasaccharides **7a** and **7b** can be converted either to **5a** (i) NaOMe-MeOH, ii) 2,2-dimethylpropane p-TsOH, iii) BnBr-NaH-DMF, iv) AcOH-H_2O, 80 ° C) or to **5b** (i)NaOMe-MeOH, ii) trimethylorthoester p-TSOH-benzene, iii) BnBr-NaH-DMF, iv) AcOH-H_2O, 80 ° C). Glycosylation of the glycotetraosyl acceptor **5a** with **3b** then gives the desired protected hexasaccharide **9a** and its (1->4) analogue **10**. On the other hand, the glycosylation of **5b** with **4b** is expected to give only one product **9b** in better yield. Each of the protected hexasaccharides **9a** or **9b** can be converted to deblocked lactonorhexaosyl **6** by the following reactions [(i) NaOMe-MeOH, ii) NH_2NH_2-H_2O-EtOH, reflux, iii) Ac_2O-MeOH, iv) 10% Pd-C, H_2].

In Figure 2, an alternative synthetic route (stepwise approach, mode II) to **6** is shown, which differs from the stepwise approach mode I (Figure 1) in the sense that two N-phthalyl lactosaminyl disaccharides can be coupled to get a tetrasaccharide and then that tetrasaccharide can be coupled to the lactosyl acceptor to give the hexasaccharide. According to this possible plan, the thioglycoside **3c** can be transformed into a lactosaminyl acceptor **12** via **11**. The tetrasaccharide thus obtained can be used as such as a donor by activating the anomeric thioalkyl group by the use of an appropriate catalyst and coupled to known lactosyl acceptor **2a** or **2b** to give the protected hexasaccharide **13**. The protected hexasaccharide can be transformed into the free **6** in the same manner as described for **9**.

Subsequently, the lactonorhexaosylceramide or analogues thereof derived from natural sources or chemically synthesized lactonorhexaosylsaccharide or analogues thereof can be converted quantitatively to their α1->3 fucosyl derivatives as shown below:



where R represents ceramide or a carrier molecule.

Any source of α1->3 fucosyltransferase can be used. Some of the preparations, depending on the source, may contain α1->4 fucosyltransferase or a non-specific enzyme that has both α1->3 and α1->4 transferase activity. However, lactonorhexaosyl and longer lactonorhexaosyl structures have no free OH group at the C4 position of GlcNAc, and therefore, the presence of α1->4 fucosyltransferase activity does not change the quality of the product, i.e., the product has exclusively α1->3 fucosyl substitution.

Other sources include human colonic mucosa and human colonic adenocarcinoma cell lines other than Colo205.

Example II describes the reaction using the enzyme from the human colonic adenocarcinoma Colo205 cell line. This cell line contains α1->3 and α1->4 fucosyltransferase, but does not contain α1->2 fucosyltransferase. Any of the other cell lines or tissues can be used if appropriate enzymatic activities are present.

In an analogous manner, structures related to the Le^x antigen can also be prepared.

For example, the Y_2 antigen can be prepared by α1->3 fucosylation at the V^3 position of the lactonorhexaosyl backbone, and the Z_1 antigen, Z_2 antigen and Z_3 antigen can be prepared by α1->3 fucosylation at the VII^3 positions only, the V^3 and VII^3 positions and the III^3, V^3 and VII^3 positions, respectively, of the lactonoroctaosyl backbone.

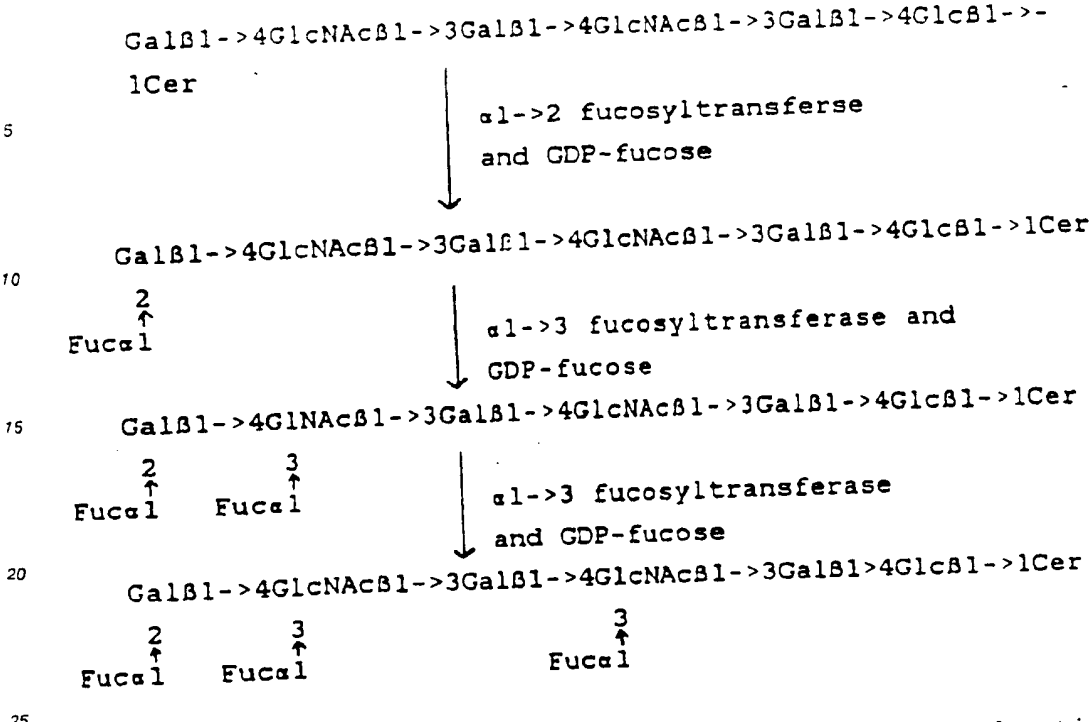
Further enzymatic synthesis could also include α1->3 fucosylation at the V^3 position and α1->2 fucosylation at the terminal galactose residue of lactonorhexaosyl backbone to give the Le^Y structure and this could be further converted to trifucosyl Le^Y by α1->3 fucosylation at the III^3 position.

Enzymatic synthesis could also include a1->4 fucosylation, etc., so long as a useful structure is produced.

The α1->3 fucotransferase and α1->4 fucotransferase enzyme preparations can be made from known sources as described above by known methods such as described in Example II. (Cf. Holmes et al, 1985, J. Biol. Chem., 260, 7619-7627).

Although actual examples of synthesis of Le^y antigen are not included in this application, Le^y antigens can be synthesized by addition of fucose at the terminal Gal residue with a1->2 fucosyltransferase and GDP-fucose followed by the action of α1->3 fucosyltransferase and GDP-fucose by analogous procedures to those followed for synthesis of dimeric Le^x antigen.

α1->2 fucotransferase capable of fucosylating a terminal galactose residue has been found in the MKN74 colonic cancer cell line although its activity is relatively weak and concentration of the enzyme activity is necessary. Since α1->2 fucosyl transferase is widely found in various cells, better sources of α1->2 fucosyl transferase are highly plausible. Thus a possibility for the following synthesis of Le^y or Le^y octasaccharide ceramide is envisioned to be within the scope of this invention.



trifucosyl Le^y antigen (important tissue-associated antigen)

GDP-fucose can be synthesized from fucose β1-phosphate by known methods (Nunez, H., O'Connor, J., Rosevear, P., Barker, R. 1981, Can. J. Chem., 59, 2086-2095; Michelson, A. 1964, Biochim. Biophys. Acta, 91, 1-13))

EXAMPLES

The present invention will now be described by reference to specific examples, but the examples are not to be construed as limiting the invention.

Unless otherwise specified, all percents, ratios, parts, etc. are by weight.

EXAMPLE 1

PREPARATION OF CORE LACTONORHEXAOSYL STRUCTURE

Five gallons of fresh bovine red blood cells were obtained from a local slaughterhouse in the form of unlysed, citrated whole blood. The five gallons were diluted to fifteen gallons by adding de-ionized H_2O at 4° C and acetic acid was added to a final concentration of 0.1%. After stirring, the red blood cells were allowed to lyse overnight at 4° C. The following morning, the lysed blood was centrifuged in a preparative rotor (Beckman JCF-Z), and the pellet was washed with H_2O. From five gallons whole blood, a 1.8 liter pellet of pinkish stroma was obtained.

1. Homogenization

The stroma pellet was homogenized in three liters of ethanol for three minutes, and then brought to slow boil in 80° C water bath for five minutes. The hot ethanol extract was filtered over a Büuchner funnel, and the residue rehomogenized in the same manner two additional times. The three filtrates were pooled and evaporated to dryness by rotovap. The organic residue was transferred to a gallon jug in three liters of chloroform- methanol (2:1, v:v), and 500 ml H_2O was added and shaken ten times to form Folch's partition. After the upper phase cleared, it was suctioned off, and the volume was replaced with chloroform-methanol-water (1:10:10, v:v:v; 0.1% KCl). After the phases separated, the second upper phase was drawn off. The two pooled upper phases were evaporated to dryness by rotovap and dialyzed in a Spectrapor (S.P.) 3 (M.W cutoff = 3500) against water for two days.

A 300 ml bed volume of DEAE Sephadex (Sigma) was used in a column 5 cm x 25 cm. The Sephadex was pre-equilibrated with chloroform-methanol-water (30:60:8, v:v:v; 0.8 M sodium acetate) overnight and washed in chloroform-methanol-water (30:60:8, v:v:v). The dialyzed upper phase was evaporated to dryness by rotovap and brought up in 500 ml chloroform-methanol-water (30:60:8, v:v:v, Sol A). This was applied to DEAE and washed with 2 liters Sol A.

The column was washed with 500 ml methanol, and the monosialoganglioside fraction (containing sialylnorhexaosylceramide) was eluted in 0.04 M ammonium acetate (1.5 liters). The monosialyl fraction was evaporated to dryness and dialyzed for three days against water, then evaporated by rotovap and transferred to a 15 ml screw cap tube.

2. Separation of Monosialyl Fraction by HPLC

A 50 cm x 1 cm column of Iatrobeads (10 µm; Iatron 6RS-8010) was preequilibrated as follows. A starting composition of 2-propanol-hexane-water (55:25:20, v:v:v) at 2.0 ml/min was applied, and a linear gradient to a final composite of 55:40:5 (v:v:v) was applied over 120 min. The sample was applied in two injections of 2 ml in chloroform-methanol-water (2:1:0.1, v:v:v) at a starting composition of 55:40:5 (v:v:v) at 0.5 ml/min. The gradient was changed to 55:25:20 (v:v:v) after 400 min and remained at this composition for a final 100 min (t = 500 min). Fractions were collected in 100 tubes, 2.5 ml/tube. Fractions were chromatographed in chloroform-methanol-water (50:40:10, v:v:v) containing 0.05% $CaCl_2$ (Merck HPTLC plates) and detected by 10% H_2SO_4 - 0.5% orcinol. The fraction containing sialyllactonorhexaosylceramide (fractions 55-58), were pooled, and further purified by rechromatography on a long Iatrobeads 6RS-8010 column (0.5 cm x 100 cm) pre-equilibrated with isopropanol-hexane-water (55:40:5, v:v:v), and the sample was applied onto the column. The column was eluted by gradient elution from isopropanol-hexane-water (55:40:3 to 55:25:20, v:v:v) over 300 min, then eluted with the same solvent for 400 min. Thus, essentially pure sialyllactonorhexaosylceramide was obtained.

The results are shown in Fig. 7.

In Fig. 7, TLC was performed by conventional methods. Fig. 7A is a thin layer chromatogram showing the elution pattern of monosialyl gangliosides through the Iatrobead column. The arrow indicates sialylnorhexaosyl ceramide. Fig. 7B shows a thin layer chromatographic pattern of fractions 51-59. Arrow 1 indicates sialyl lactonorooctaosylceramide, and arrow 2 indicates sialyl lactoisooctaosylceramide. These fractions are the major source for the preparation of lactonorhexaosylceramide (Galβ1->4GlcNAcB1->3Galβ1>4-GlcNAcβ1->3Galβ1->4Glcβ1->1Cer) and lactoisooctaosylceramide (I antigen), respectively. Fig. 7C shows the thin layer chromatographic pattern of several pooled fractions. The pooled fraction (55-58) in Fig. 7C was subjected to a second HPLC, and the separation pattern is shown in Fig. 7D.

3. Hydrolysis with Acetic Acid

2 mg of sialyllactonorhexaosylceramide was evaporated to dryness in an 8 ml screw cap tube, and 1 ml of 1.0% acetic acid added. The tube was placed in a 100° C heat block for 1 hr. After hydrolysis, the sample was evaporated to dryness by adding 2 ml ethanol and drying under an N_2 stream. The dried glycolipid was dissolved in 2 ml of water and applied to a Sep-Pak C-18 reverse phase column, washed intensively with water, and eluted with methanol. The eluate was evaporated. The reaction was quantitative.

EXAMPLE II

ENZYMATIC α1->3 FUCOSYLATION

50 g of solid tumors in nude mice (40-50 tumors combined) were homogenized on ice in 100 ml of 50mM Tris buffer pH 7.4, 1% Triton X-100, 2mM $MnCl_2$, and 25% glycerol in a Brinkman Homogenizer for 3 x 30 seconds at 100,000 xg. This supernatant was used as the enzyme source (Preparation I).

GDP-fucose, the sugar donor, is commercially available or can be produced from fucose β1-phosphate (Nunez, H., O'Conner, J., Rosevear, P., Barker, R., 1981, Can. J. Chem., 59, 2086-2095; Michelson, A., 1964, Biochem. Biophys. Acta, 91, 1-13).

The enzymatic reaction was carried out in various scales. The following description indicates one example. 10 mg of lactonorhexaosylceramide (nLc_6) or lactonorhexaosyl oligosaccharide was mixed with 2 mg of sodium taurocholate in a 100 ml round bottom flask and co-dissolved in a suitable solvent, thoroughly mixed and evaporated to dryness. The dried residue was dissolved in 1 ml water, 50 µl of 0.4 M $MnCl_2$, and 500 µl of 1.0 M Tris buffer pH 7.4, and sonicated well, and 500 µl of aqueous solution containing 50 mg CDP-choline (Sigma) and 10 mg GDP-fucose.

The total mixture was then added to 10 ml of the enzyme "Preparation I". The reaction then proceeded at 37° C with shaking. After 18 hr, 100 mg CDP-choline and 20 mg GDP fucose in 1 ml H_2O were added, and the reaction was left another 24 hrs.

The entire reaction mixture, when starting from lactonorhexaosylceramide, was then applied to a C18 HPLC column and washed with water extensively to eliminate all water-soluble components in the reaction mixture. Glycolipids were eluted from the column with methanol followed by chloroform-methanol (2:1, v:v), then further purified on HPLC with an isopropanol-hexane-water system by known methods.

The reaction mixture, when starting from lactonorhexaosyl oligosaccharide linked to the carrier peptide or protein, was placed on an affinity column containing SH2 antibody, which reacts with dimeric Le^x. The compound was diluted with 1-2 M NaCl. Under these conditions, about 60-70% of the norhexaosyl structure was converted to dimeric Le^x, based on orcinol detection of resultant glycolipid product. TLC plate was chromatographed and detected by 0.5% oricinol in 10% H_2SO_4, heated at 150° C for 3 minutes. Percentage conversion was based upon densitometer readings giving area units for each band. About 10% was not converted, and about 20-30% was converted to monofucosyl derivative.

Summary of Enzymatic α1->3 Fucosylation

1. Enzyme Sources:

a) Human Colonic Mucosa
b) Human Colonic Adenocarcinoma Cell Lines Colo205, human intestine, etc.

Crude homogenate
Golgi-enriched fraction
Detergent soluble fraction
Semi-purified (ion exchange, GDP hexanolamine, etc.)

50 gm solid tumors grown in nude mice (40-50 tumors). Place in 100 ml of 50 mµ $MnCl_2$ and 25% glycerol homogenized in Brinkman 3 x 30 min on ice.

↓

Spin 100,000 g
Supernatant is enzyme prep I.

Reaction was allowed to proceed overnight at 37° C with shaking. After 18 hours, CDP-choline and GDP-fucose were added (Doubling amount added in each case) and reaction left another 24 hours. At this point about 60-70% of nLc_6 had been converted to dimeric Le^x. About 10% was unconverted and about 20-30% was converted to mono-fucose glycolipid.

2. Nucleotide Sugar:

GDP-fucose can be obtained commercially or produced from fucose β1-phosphate as described above.

3. Reaction Mixture:

10 mg of nLc_6 from human placenta (100% pure) was dried with 2 mg taurocholate 100 ml round bottom by rotovap. To this was added:
500 µl of 0.4 m $MnCl_2$ (200 µ moles)
500 µl of 1.0 m Tris pH 7.4
1.0 ml H_2O sonicated and mixed.

↓

Add 50 mg CDP-choline in 500 µl H_2O
10 mg GDP-fucose (Biocarb) in 530 µl H_2O

↓

Plus 10 mls of Colo205 tumor enzyme prep I.

Various assays were then performed in order to characterize the reaction products.

The results are shown in Figures 8, 9 and 10.

In Figs. 8, 9 and 10, TLC was carried out according to known methods, as was orcinal staining and immunostaining with SH1 and SH2.

In order to obtain the autoradiograms in Fig. 9, reaction mixtures were spotted at 2000 cpm/lane and developed for 18 hours on film.

Figure 8 shows immunostained thin layer chromatographs using primary antibodies as shown, secondary rabbit anti-mouse antibodies, and finally iodinated ^{125}I-protein A according to known methods, of enzymatically synthesized dimeric Le^x. Figure 8A shows the pattern after staining with 0.5% orcinol, 10% H_2SO_4 by known methods. Orcinol staining detects carbohydrate. Fig. 8B shows the TLC pattern after immunostaining with monoclonal antibody SH1, detecting long and short chain Le^x glycolipid; Fig. 8C shows the TLC pattern after immunostaining with SH_2 which detects only long chain Le^x (dimeric Le^x). Lane 1, nLc_6 standard from bovine RBC; lane 2, enzyme reaction mixture without nLc_6 (the fast migrating band is sodium deoxycholate, the lower band is derived from impurity in the enzyme preparation); lane 3, enzyme reaction mixture with nLc_6; lane 4, standard dimeric Le^x from human tumor; lane 5, standard "0" upper neutral from human RBC; lane 6, tumor upper neutral. Note conversion of nLc_6 to dimeric Le^x in lane 3 as seen by orcinol detection (Fig. 8A) as well as immunostaining with anti-Le^x (Figs. 8B and 8C).

Figure 9 is an audioradiogram showing the results of GDP-^{14}C-fucose incorporation into nLc_4 and nLc_6 from bovine and human placenta lactosyl substrates. Lane A, bovine RBC nLc_4 and GDP-^{14}C-fucose enzyme reaction mixture; Lane B, human placenta nLc_4 and GDP-^{14}C-fucose enzyme reaction mixture; Lane C, bovine RBC nLc_6 and GDP-^{14}C-fucose enzyme reaction mixture; Lane D, human placenta nLc_6 and GDP-^{14}C-fucose enzyme reaction mixture. The appearance of two doublets was noted in conversion of nLc_6 denoting the addition of one and two fucose residues, respectively. The intermediate product, with only one fucose added, is not SH1-active (Figures 10B and 10C, lane 3), and therefore, fucose is added internally and not at the terminal G1cNac (i.e., Le^x epitope).

Figure 10 shows TLC immunostaining of nLc_4 conversion of Le^x. Fig. 10A shows detection with orcinol-H_2SO_4 as in Figure 8A. Fig. 10B shows the immunostaining pattern with anti-Le^x antibody SH1. Fig. 10C shows immunostaining with anti-dimeric Le^x antibody SH2. Lane 1, standard "0" upper neutral; lane 2, tumor upper neutral; lane 3, tumor Le^x standard; lane 4, enzyme reaction mixture with nLc_4 from human placenta; lane 5, enzyme reaction mixture with nLc_4 from bovine RBC. Fig. 10B, lanes 4 and 5 show SH1-positive reaction demonstrating production of the Le^x epitope. SH2 will not stain short-chain Le^x.

As already discussed above, many of the fucosylated structures produced according to the present

invention are useful as active vaccines for tumors. The skilled artisan can readily determine which structures are useful for which tumors. Further suitable pharmaceutically acceptable carriers, diluents or excipients for the vaccine and suitable methods of administration and doses can readily be determined by the skilled artisan.

Also dimeric Le^x has been shown to be useful to suppress the appearance of inflammatory granulocytes in the bone marrow of RA-affected joints.

Further, many of the structures will be useful for proucing monoclonal antibodies with specific binding for that substance. Such monoclonal antibodies can be used for passive immunization against tumors and for in vitro and in vivo methods for detecting tumors. Such methods are readily known to and conducted by the skilled artisan.

EXAMPLE III

SYNTHESIS OF Le^y ANTIGENS

A series of Le^yantigens can be prepared by a similar enzymatic fucosylation as that applied for synthesis of dimeric Le^x described above. Lactonorhexaosylceramide or lactonorhexaosyl saccharides linked to carrier molecules or their higher analogues are the starting material. The material is dissolved in Tris buffer in the presence of bivalent cation and appropriate detergent such as taurocholate, as described above. To this solution is added GDP-fucose, CDP-choline, and α1->2 fucosyltransferase, which is isolated from human gastric cancer cell line MKN74 or the same enzyme isolated from any other source. The reaction can be similarly processed as described above, and subsequently α1->2 fucosylated derivatives will be isolated, which can be monitored by positive reaction with monoclonal antibody BE2 (Young WW et al., 1981, J. Biol. Chem., 256, 10967-10972). The H-active compound as an intermediate can be further processed for α1->3 fucosylation by α1->3 fucosyltransferase of Colo205 and GDP-fucose under the same conditions as described above for dimeric Le^x. The final product can be monitored by positive reaction with monoclonal antibody AH6 defining Le^ystructure (Abe K et al, 1983, J. Biol. Chem., 258, 11793-11797) and monoclonal antibody directed to trifucosyl $Le^y$and extended Le^y(Kaizu et al, 1983, J. Biol. Chem., 261, 11254-11258).

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Claims

1. A process for preparing difucosyl Y_2 antigen (dimeric Le^x), said process comprising:

(1) preparing a lactonorhexaosylceramide backbone or a lactonorhexaosylsaccharide backbone linked to a carrier molecule; and

(2) enzymatically fucosylating said backbone at the III^3 and V^3 positions through an α1->3 linkage.

2. The process of Claim 1, wherein said backbone is prepared by organic chemical synthesis.

3. The process of Claim 1, wherein said backbone is prepared by isolation from a natural source.

4. The process of Claim 3, wherein said natural source is bovine red blood cells, human placenta or rabbit muscle.

5. The process of Claim 1, wherein said step (2) of enzymatically fucosylating said backbone is performed with α1->3 fucosyltransferase isolated from human colonic adenocarcinoma Colo205 cell line in the presence of GDP-fucose.

6. A process for preparing Le^y antigen analogues, said process comprising:

(1) preparing a lactonorhexaosylceramide backbone or a lactonorhexaosylsaccharide backbone linked to a carrier molecule;

(2) enzymatically fucosylating said backbone at the terminal β-Gal through an α1->2 linkage; and

(3) enzymatically fucosylating said backbone at one or more positions through an α1->3 linkage, provided that steps (2) and (3) can be conducted simultaneously or in any order.

7. The process of Claim 6, wherein said step (2) is conducted before said step (3).

8. The process of Claim 6, wherein said backbone is prepared by organic chemical synthesis.

9. The process of Claim 6, wherein said backbone is prepared by isolation from a natural source.

10. The process of Claim 9, wherein said natural source is bovine red blood cells, human placenta or rabbit muscle.

11. The process of Claim 6, wherein said step (2) of enzymatically fucosylating said backbone at the terminal β-Gal is performed with α1->2 fucosyltransferase in the presence of GDP-fucose.

12. The process of Claim 6 or Claim 11, wherein said step (3) of enzymatically fucosylating said backbone is performed with α1->3 fucosyltransferase isolated from human colonic adenocarcinoma Colo205 cell line in the presence of GDP-fucose.

13. The process of Claim 6, wherein said Le^y antigen analogues are Le^y octasaccharide ceramide or trifucosyl Le^y.

14. A process for preparing an α1->2 and/or α1->3 fucosylated lactonorhexaosylceramide, lactonorhexaosylsaccharide linked to a carrier molecule or higher analogues thereof, said process comprising:

(1) preparing a lactonorhexaosylceramide backbone, a lactonorhexaosylsaccharide backbone linked to a carrier molecule or backbones of higher analogues thereof; and

(2) enzymatically fucosylating one or more residues of said backbone.

15. The process of Claim 14, wherein said step (2) comprises enzymatically glycosylating said backbone at one or more internal N-acetylglucoseamine residues through an α1->3 linkage.

16. The process of Claim 14 or 15, wherein said backbone is prepared by organic chemical synthesis.

17. The process of Claim 14 or 15, wherein said backbone is prepared by isolation from a natural source.

18. The process of Claim 17, wherein said natrual source is bovine red blood cells, human placenta or rabbit muscle.

19. The process of Claim 14 or 15, wherein said step (2) of enzymatically fucosylating said backbone is performed with α1->3 fucosyltransferase isolated from human colonic adenocarcinoma Colo205 cell line in the presence of GDP-fucose.

20. The process of Claim 14 or 15, wherein said fucosylated lactonorhexaosylceramide, lactonorhexaosysaccharide linked to a carrier molecule or higher analogues thereof is selected from the group consisting of Y_2 antigen, Z_1 antigen, Z_2 antigen and Z_3 antigen (trimeric Le^x).

FIG. 1 Synthetic Plan for Lactonorhexaosyl (Structure 6) by a Stepwise Approach (Mode I)

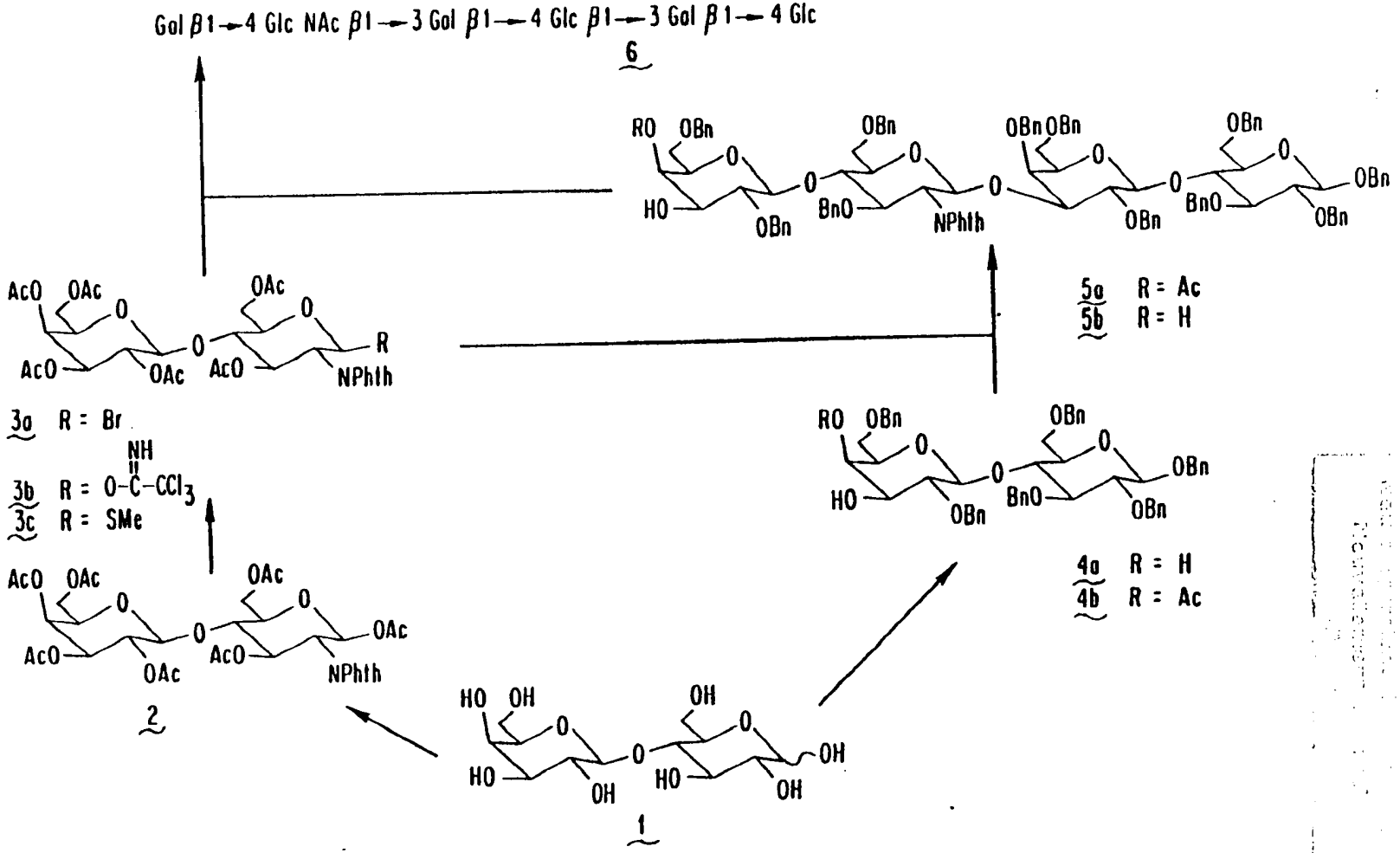


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FIG.2 Synthetic Plan for Lactonorhexaosyl (Structure 6) by a Stepwise Approach (Mode II)

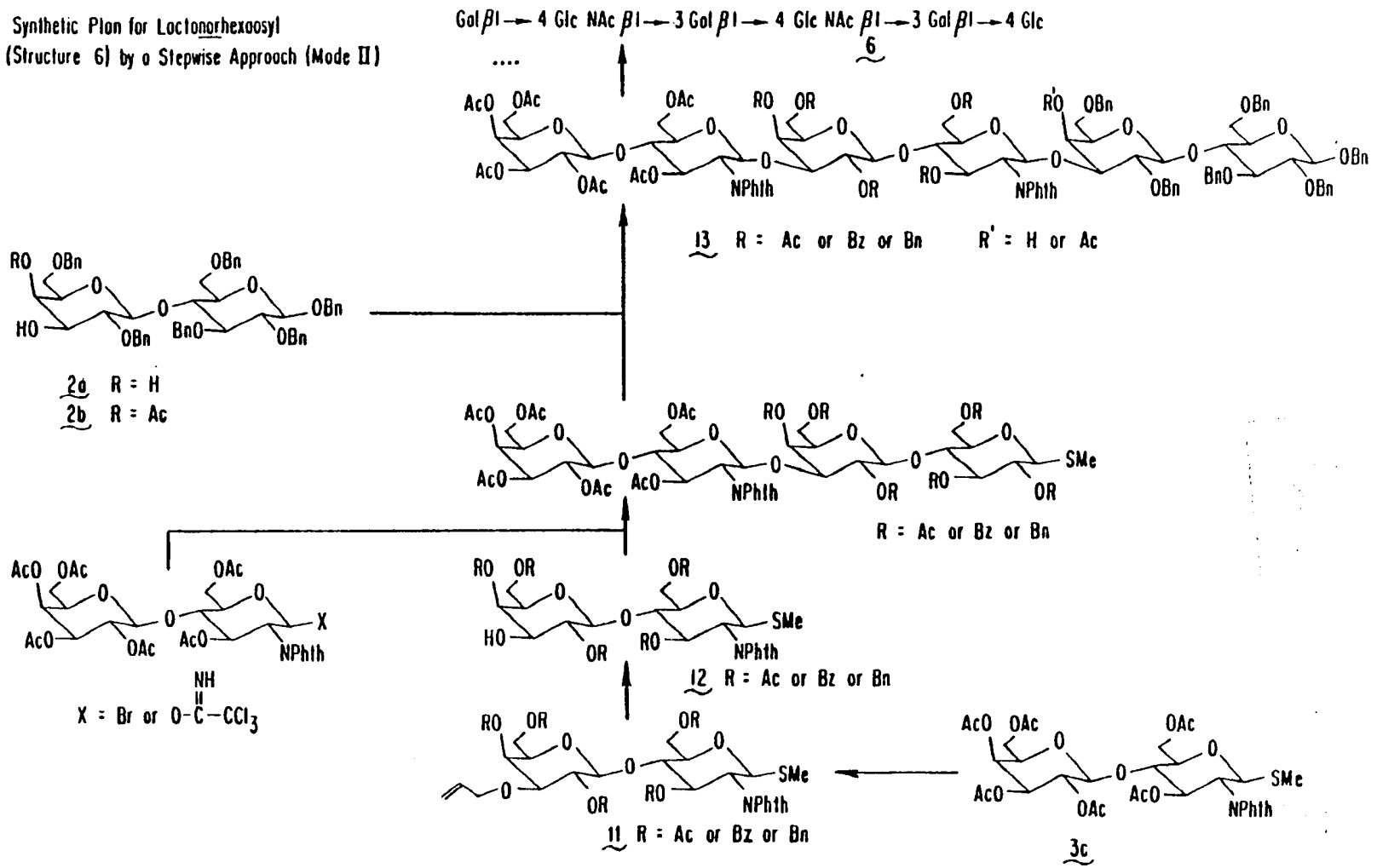

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FIG.3 PRIOR ART

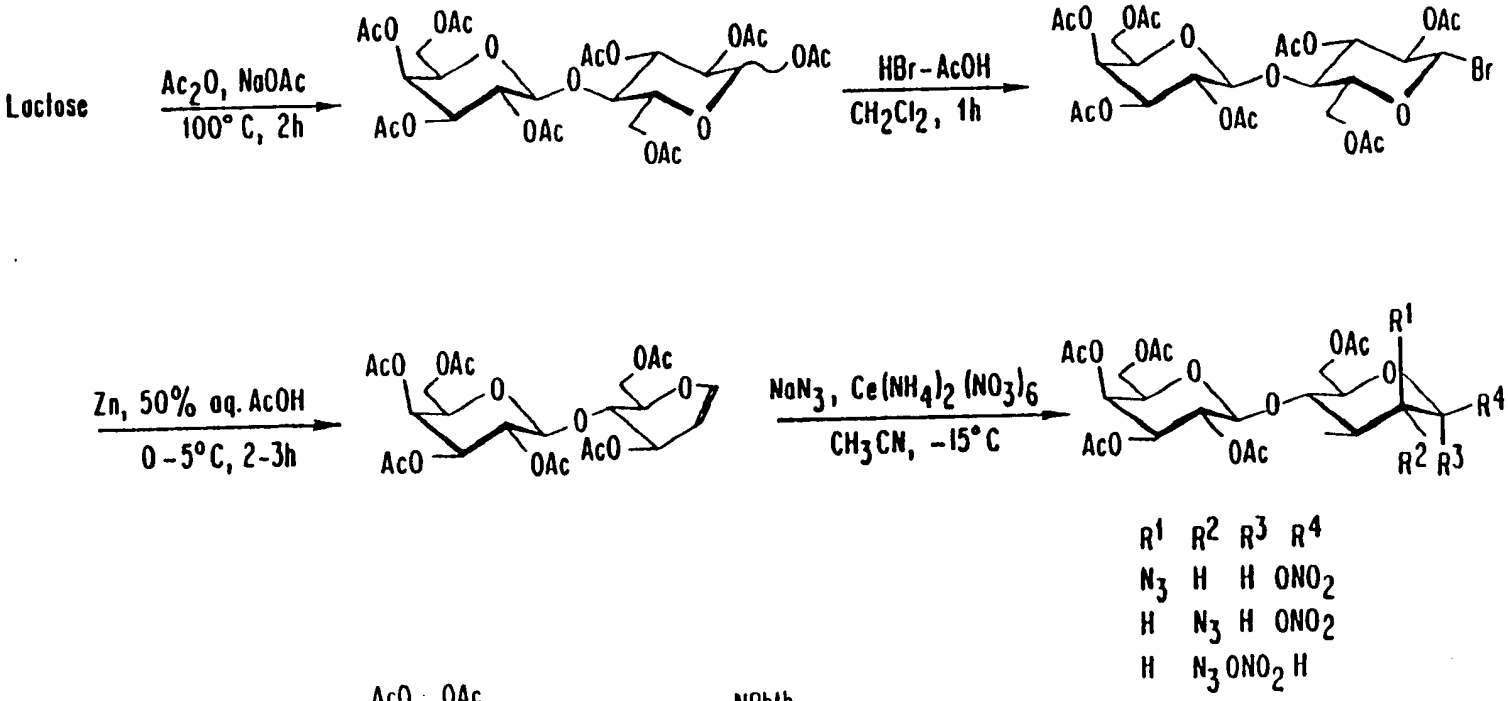

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FIG.4 PRIOR ART

Synthesis of Lactosaminyl donors

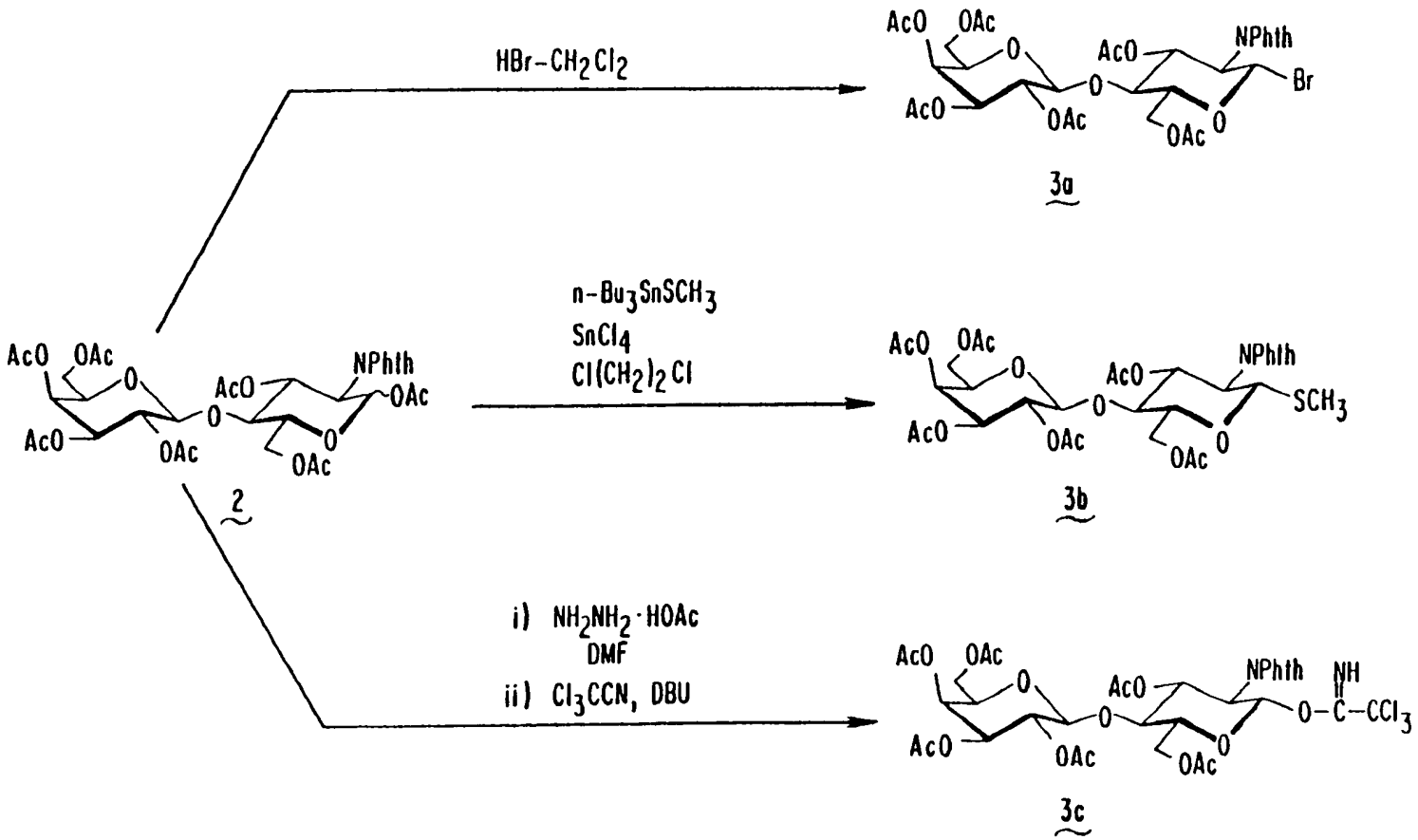

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FIG.5 PRIOR ART

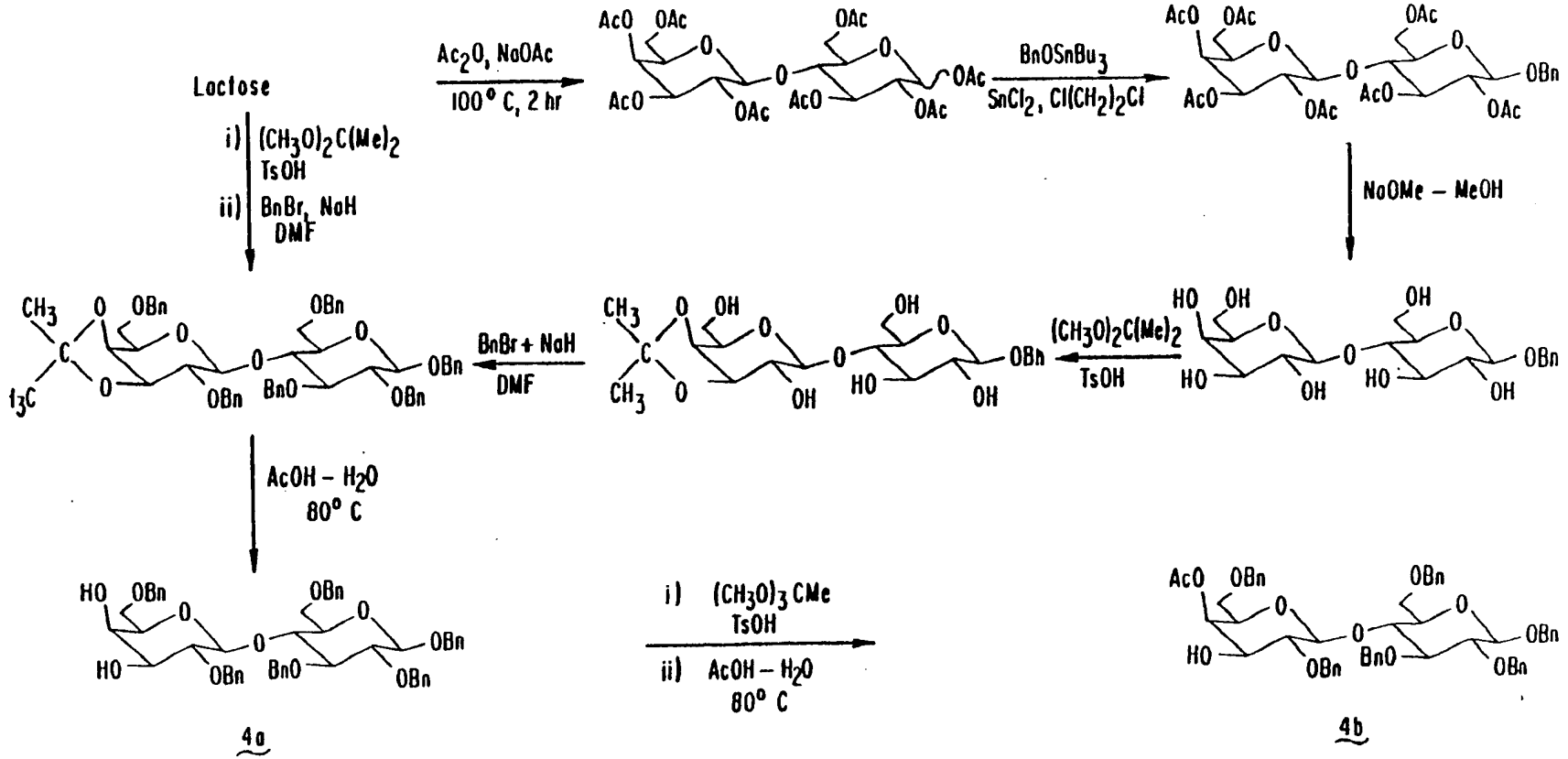

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FIG. 6 Synthesis of Loctonorhexosyl (Structure 6)

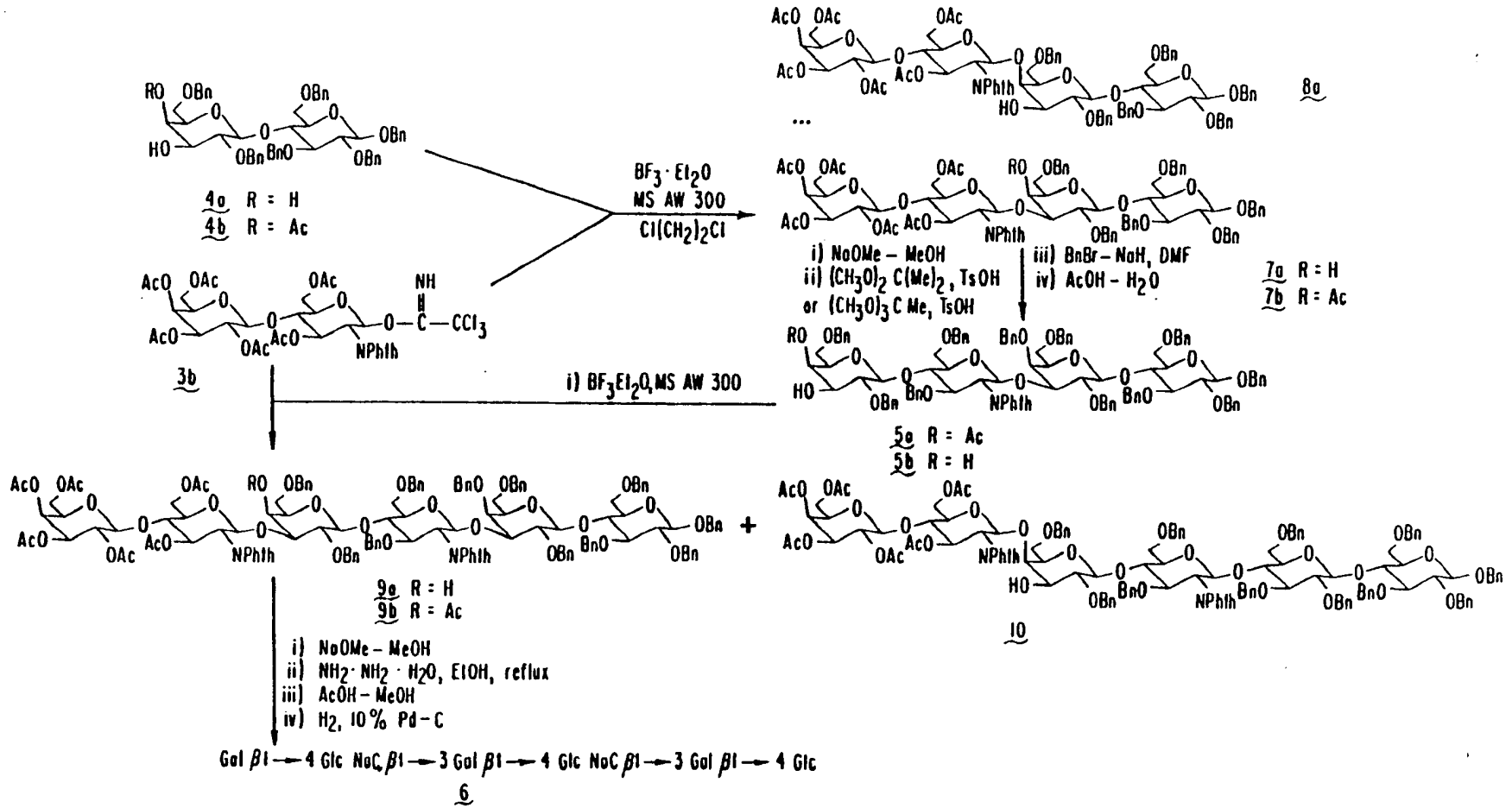

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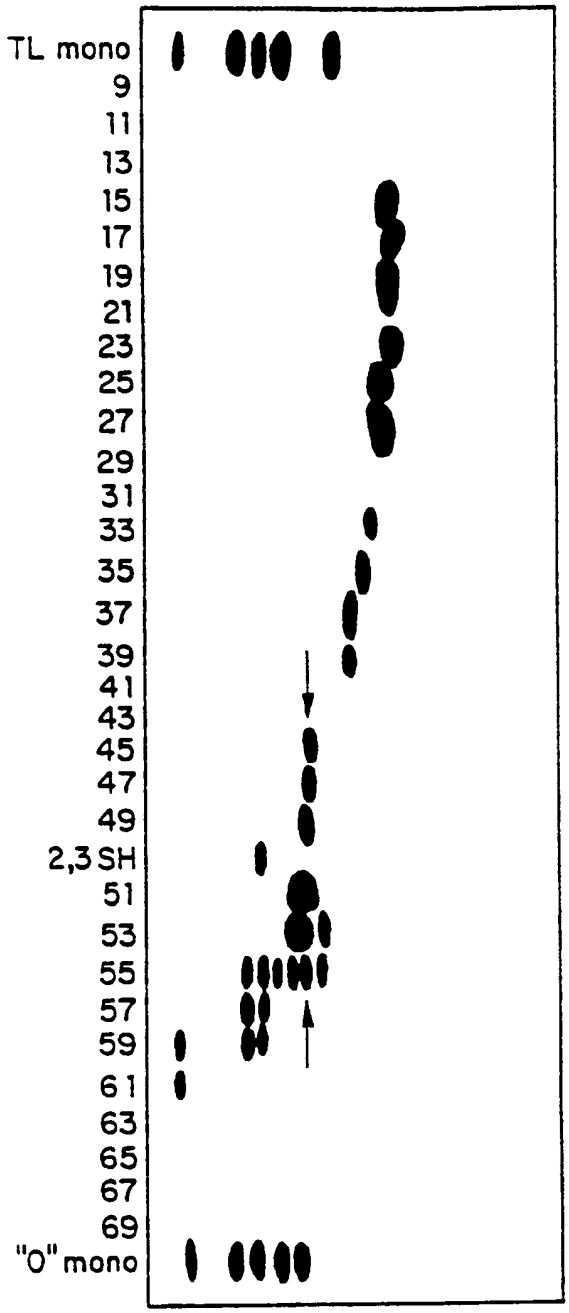
FIG.7A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2,3 SH
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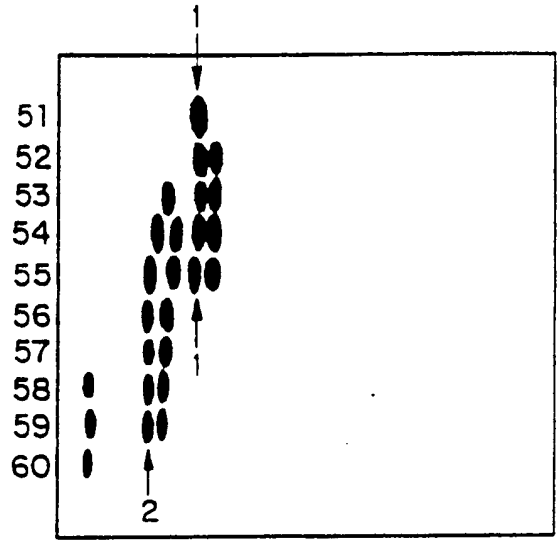
67
69
"0" mono

FIG.7B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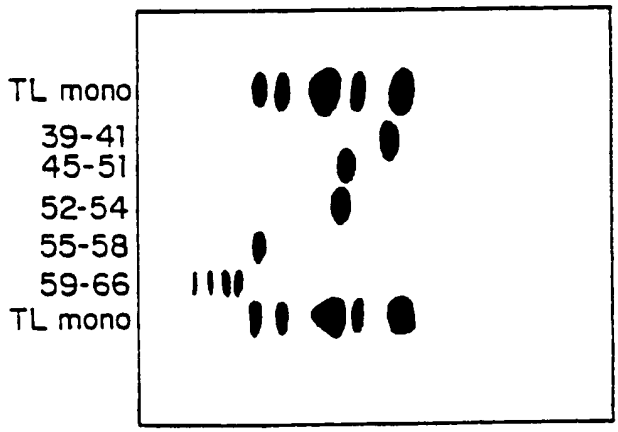
2

FIG.7C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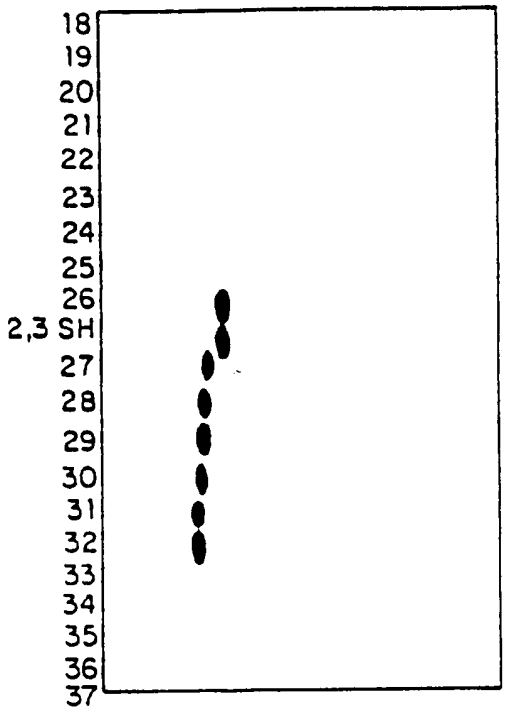


FIG.7D

FIG.8A FIG.8B FIG.8C

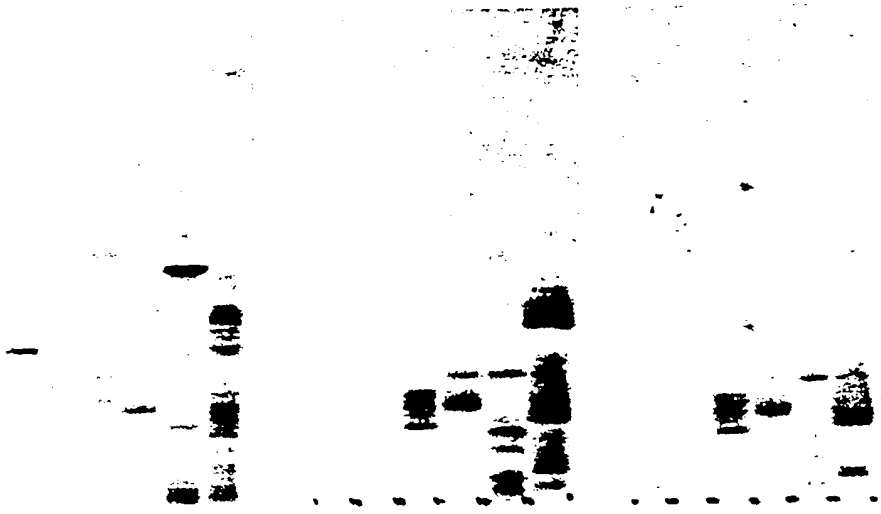


FIG.9

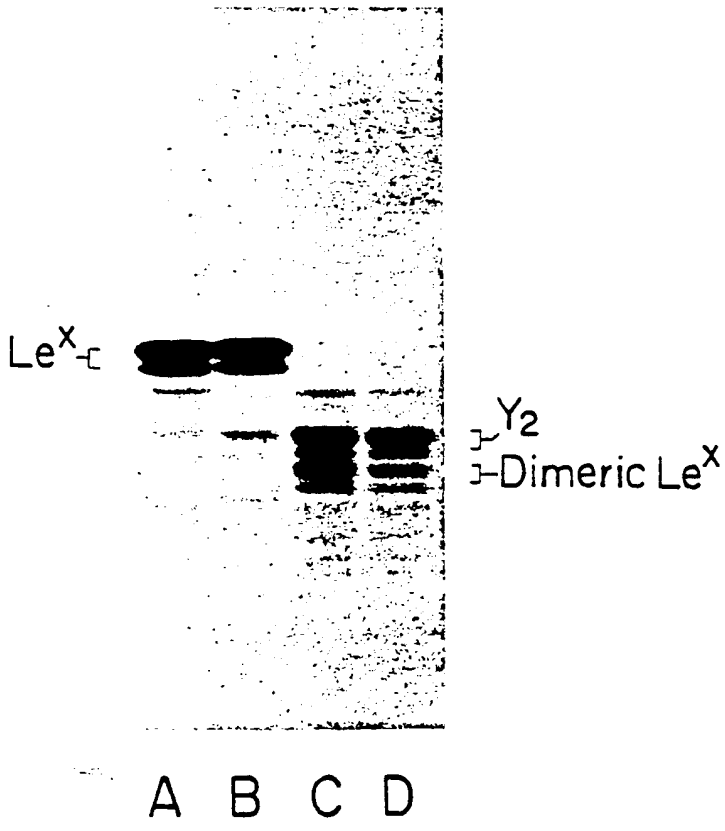
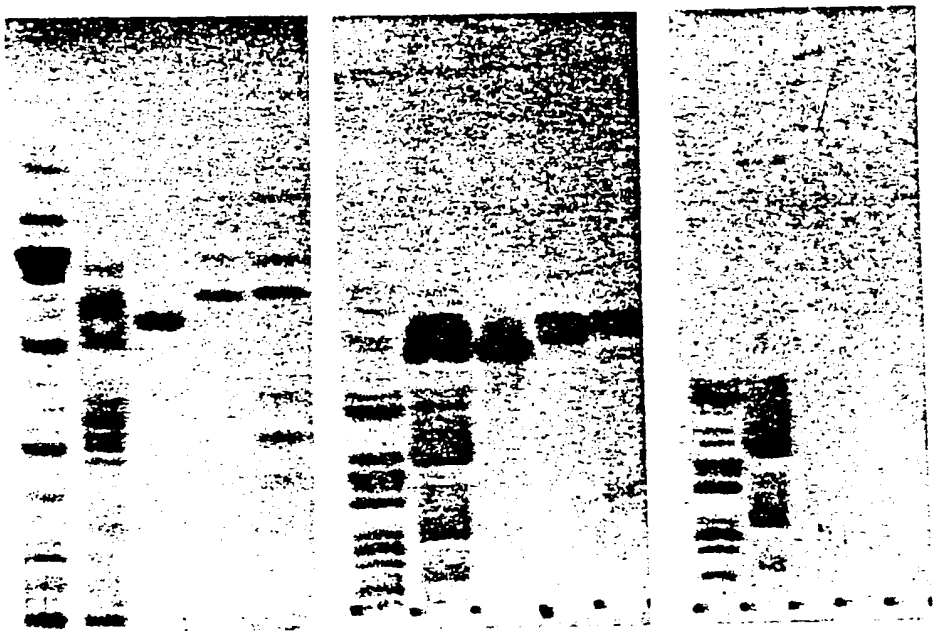


FIG.10A FIG.10B FIG.10C



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(54) Bio-organic synthesis of dimeric LeX (difucosyl Y2; III3FucV3-FuncnLc6Cer) and analogues thereof.

(57) A process for preparing difucosyl Y_2 antigen (dimeric Le^x), said process comprising: (1) preparing a lacto nor hexaosylceramide backbone or a lacto nor hexaosylsaccharide backbone linked to a carrier molecule; and (2) enzymatically fucosylating said backbone at the III^3 and V^3 positions through an α1->3 linkage. A process for preparing Le^y antigen analogues, said process comprising: (1) preparing a lacto nor hexaosylceramide backbone or a lacto nor hexaosylsaccharide backbone linked to a carrier molecule; (2) enzymatically fucosylating said backbone at the terminal β-Gal through an α1->2 linkage; and (3) enzymatically fucosylating said backbone at one or more positions through an α1->3 linkage, provided that steps (2) and (3) can be conducted simultaneously or in any order. A process for preparing a fucosylated lacto nor hexaosylceramide, lacto nor hexaosylsaccharide linked to a carrier molecule or higher analogues thereof, said process comprising: (1) preparing a lacto nor hexaosylceramide backbone, a lacto nor hexaosylsaccharide backbone linked to a carrier molecule or backbones of higher analogues thereof; and (2) enzymatically fucosylating one or more residues of said backbone.

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90303034.4

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,A	TETRAHEDRON LETTERS, vol. 29, no. 41, 1988 S. SATO et al. "A total synthesis of dimeric Lex Antigen, III3V3Fuc2nLc6Cer: Pivaloyl auxiliary for stereocontrolled glycosylation" pages 5267-5270 * Abstract * --	1	C 12 P 19/04
D,A	CARBOHYDRATE RESEARCH, vol. 183, 1988 M. NISSON AND T. NORBERG "Synthesis of a dimeric Lewis X hexasaccharid derivative corresponding to a tumor--associated glycolipid" pages 71-82 * Abstract * --	1	
D,A	GLYCOCONJUGATE JOURNAL, vol. 4, no. 1, 1987 M. NILSSON AND T. NORBERG "Synthesis of a dimeric lewis-x Hexasaccharid as a p-trifluoroacetamido--phenylethyl ß-Glycoside" pages 219-223 * Totality * ----	1	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	12-11-1990	SCHNASS

CATEGORY OF CITED DOCUMENTS

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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

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